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(71) Applicants (for all designated States except US): TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA [US/US]; Suite 300, 3700 Market Street, Philadelphia, PA 19104–3147 (US). NATIONAL INSTITUTE OF HEALTH [US/US]; Suite 325, 6011 Executive Boulevard, Rockville, MD 20852 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): LIANG, Bruce, T. [US/US]; 405 Merwyn Road, Merion Station, PA 19066 (US). JACOBSON, Kenneth, A. [US/US]; Apartment 504, 1111 University Boulevard, Silver Spring, MD 20902 (US).

(74) Agents: RIGAUT, Kathleen, D. et al.; Dann, Dorfman, Herrell and Skillman, Suite 720, 1601 Market Street, Philadelphia, PA 19103–2307 (US).

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(54) Title: METHODS FOR REDUCING ISCHEMIC INJURY OF THE HEART VIA THE SEQUENTIAL ADMINISTRATION OF MONOPHOSPHORYL LIPID A AND ADENOSINE RECEPTOR AGENTS

(57) Abstract

Materials and methods for reducing or preventing ischemic damage of the heart are disclosed. A preferred embodiment of the invention comprises methods of sequential administration of a plurality of cardioprotective agents to patients suffering from ischemic damage or at risk for the same.

METHODS FOR REDUCING ISCHEMIC INJURY OF THE HEART VIA THE SEQUENTIAL ADMINISTRATION OF MONOPHOSPHORYL LIPID A AND ADENOSINE RECEPTOR AGENTS

10   **FIELD OF THE INVENTION**

The present invention relates to methods for administering compounds to protect the heart from ischemic injury. More specifically, the invention provides a novel combination of agents which act

15   synergistically to potentiate their individual cardioprotective effects and thereby render the myocardium more resistant to ischemia.

BACKGROUND OF THE INVENTION

Several publications are referenced in this

20   application by numerals in parentheses in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated

25   by reference herein.

Ischemic preconditioning functions as an endogenous protective mechanism which enhances the ability of myocardium to withstand injury from prologed ischemia. Transient ischemic events, which are sufficiently brief

30   to avoid irreversibly damaging myocardium, initiate unidentified biochemical events which limit infarct size and the occurrence of reperfusion arrhythmias following prolonged myocardial ischemias (5).

A series of studies have shown that it is possible

35   to induce ischemic preconditioning with a variety of pharmacological agents. Adenosine is released in large

amounts during myocardial ischemia and mediates potentially important protective functions in the cardiovascular system (1,4,5,7,9,14,17,18,19,25). Adenosine can precondition the heart with reduction in

5   the size of myocardial infarction (4,5,9,14,17,18). Intracoronary administration of adenosine during reperfusion following prolonged no-flow ischemia can also limit infarct size in the intact heart (1, 19).

Previous studies have shown that adenosine A_1 and A_3

10   receptor agonists can precondition the heart when administered before the onset of ischemia (4, 5, 9, 14, 17, 18). Other studies have shown that adenosine A_{2a} receptor antagonists also enhance the cardioprotective effect of preconditioning (23). These agents effectively

15   1) reduce infarct size; and 2) improve left ventricular function when given during reperfusion (1, 19) or during both low-flow ischemia and reperfusion in isolated perfused heart (6, 21, 22).

Monophosphoryl lipid A (MLA), a relatively non-toxic

20   derivative of endotoxin, has also been found to provide cardioprotection in a variety of animal models (43-45) when administered prior to an ischemic event. The mechanism of myocardial protection mediated by MLA has not yet been definitively elucidated. Other effective

25   pharmacological preconditioning agents include K^+ATP channel openers and phorbol esters.

Intensive research efforts are currently focused on the development of agents and methods for treating and preventing cardiac diseases. The present invention is

30   directed to such methods.

SUMMARY OF THE INVENTION

Methods are disclosed which may be used to advantage for preventing ischemic damage of the heart.

The methods of the invention entail the combined

35   administration of a first cardioprotective agent and a second cardioprotective agent. The agents act

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synergistically to potentiate their individual
cardioprotective effects. Agents suitable for use as a
first cardioprotective agent are adenosine receptor
agents, MLA or analogs or derivatives of MLA.

5   According to a preferred embodiment, the invention
entails the administration of monophosphoryl lipid A
(MLA), or a synthetic analog, or derivative thereof to a
patient prior to a surgical treatment. Following MLA
treatment, a second cardioprotective agent is

10   administered that potentiates the cardioprotective effect
of MLA. The second cardioprotective agent is delivered
to the patient either before, during or after surgery.
Alternatively, the second cardioprotective agent may be
administered continuously throughout all of these

15   periods. The protocols described above may be used for
the treatment of patients at risk for ischemic damage
from myocardial infarction, angina, or surgical
complications.

       Compounds suitable as second cardioprotective agents

20   are adenosine, agonists at the A_1 and A_3 adenosine
receptors, antagonists at the A_{2a} adenosine receptor, and
K_{ATP} channel openers.

       In an alternative embodiment, an adenosine receptor
agonist is administered as the first cardioprotective

25   agent and MLA or synthetic analogs thereof is
administered as the second cardioprotective agent.

       Methods of administration of the cardioprotective
agents of the invention include direct perfusion of the
organ during surgery and intravenous administration.

30   Additionally, these agents may be administered to
patients in solid form, e.g., tablets, in amounts
effective to prevent or reduce ischemic damage to the
heart.

BRIEF DESCRIPTION OF THE DRAWINGS

35   Figure 1 is a graph showing the protective effect of
pretreatment with MLA prior to prolonged ischemia in

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cardiac myocytes.

Figure 2 is a graph showing that pretreatment with MLA enhances the cardioprotective effect of preconditioning ischemia in cardiac myocytes.

5   Figure 3A is a graph showing that pretreatment with MLA enhances the preconditioning effect mediated by adenosine in cultured myocytes ($\square$, vehicle; $\bigcirc$, MLA 30 ng/ml; $\blacksquare$, MLA, 300 ng/ml). Cardiac ventricular myocytes were subjected to classical preconditioning as described

10   herein. Figures 3B and 3C are graphs showing the results obtained when myocytes were treated with CCPA (Fig. 3B) or IB-MECA (Fig. 3C) for five minutes followed by incubation in adenosine- or agonist-free media for 10 minutes prior to 90 minutes of ischemia. Data, plotted

15   as percentage of cells killed and the amount of CK released, represented the mean and standard error of four experiments. * indicates significant difference from vehicle-treated myocytes at the concentrations of adenosine agonists indicated (t test, $P<0.001$).

20   Figure 4 is a graph showing that pretreatment with MLA enhances the preconditioning effect mediated by phorbol 12-myristate 13-acetate (PMA)($\square$, vehicle; $\blacksquare$, MLA 300 ng/ml).

Figure 5 is a graph showing that pretreatment with
25   MLA enhances the preconditioning effect mediated by pinacidil in cardiac myocytes ($\square$, vehicle; $\bigcirc$, MLA 30 ng/ml; $\blacksquare$, MLA, 300 ng/ml).

Figure 6 is a graph showing that pretreatment with MLA enhances the cardioprotective effect of the A_1
30   adenosine receptor agonist, 2-chloro-N^6-cyclopentyl-adenosine (CCPA), during prolonged ischemia ($\square$, vehicle; $\blacksquare$, MLA 300 ng/ml).

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Figure 7 is a graph showing that pretreatment with
MLA enhances the cardioprotective effect of the A_3
adenosine receptor agonist, N^6-(3-iodobenzyl)adenosine-5'-
N-methyluronamide (IB-MECA), during prolonged ischemia
5   ($\square$, vehicle; $\blacksquare$, MLA 300 ng/ml).

Figure 8 is a graph showing that pretreatment with
increasing concentrations of MLA enhances the
cardioprotective effect of pinacidil during prolonged
ischemia ($\square$, vehicle; $\blacksquare$, MLA 300 ng/ml).

10   **DETAILED DESCRIPTION OF THE INVENTION**
Preconditioning with brief ischemia before a
sustained period of ischemia reduces infarct size in the
perfused heart. Ventricular myocytes cultured from chick
embryos retain many of the properties of the intact
15   heart, which make them a useful model for a variety of
experimental paradigms (33-39), including preconditioning
(16, 23, 32). This model system has been utilized to
develop novel combinations of pharmcological agents which
act synergistically to treat and prevent ischemic damage
20   of the heart.
Monophosphoryl lipid A (MLA), a derivative of the
gram-negative bacterial cell wall component
lipopolysaccharide A (i.e. endotoxin), induces a
glibenclamide-sensitive cardioprotective effect in
25   isolated, intact heart models of ischemia-reperfusion.
The data reveal that treatment with MLA has a prolonged
protective effect (31). The present invention is based
on the hypothesis that MLA or MLA analogs effect
cardioprotective action by enhancing the sensitivity of
30   the cardiac myocytes to preconditioning stimuli and to
cardioprotective agents. These cardioprotective agents
include agonists at the A_1 and A_3 adenosine receptors,
antagonists at the A_{2a} adenosine receptor, K_{ATP} channel
openers and phorbol esters. MLA may also enhance the

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protective effects of these agents during prolonged, infarct-producing ischemia. These two novel cardioprotective mechanisms of MLA were investigated and the results are provided hereinbelow.

5   The present invention demonstrates that pretreatment of myocytes with MLA alone prior to preconditioning ischemia can enhance the cardioprotective effect of the preconditioning, thus further decreasing myocyte killing in response to an ischemic event.

10   Furthermore, a synergistic cardioprotective effect is achieved by treatment with MLA in conjunction with adenosine, agonists of the A_1 and A_3 adenosine receptors, antagonists of the A_{2a} adenosine receptor or a K_{ATP} channel opening compound.

15   The compounds of the present invention tend to produce certain unwanted side effects when administered individually at standard dosage. Because these compounds have been discovered to act synergistically when used in combination, subthreshold concentrations may be employed

20   in practicing the methods of the present invention. These subthreshold concentrations should induce fewer side effects and accordingly constitute an improvement in currently available methods for reducing ischemic damage to the heart.

25   The following definitions are provided to facilitate understanding of the present invention.
Preconditioning ischemia - A brief ischemia which does not cause any cardiac damage, but protects the heart against damage during a subsequent prolonged ischemia.

30   The effect of preconditioning ischemia is mediated by adenosine, which is released during the ischemia. Preconditioning may be induced by brief exposure to anoxic conditions for example.
Adenosine receptors - A_1, A_3 and A_{2a} receptors are present

35   on the myocardium (cardiac muscle cells). While activation of the A_1 and A_3 receptors is cardioprotective, activation of the A_{2a} receptors is deleterious and causes

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damage to the cardiac muscle cells.

Stable angina – Condition observed in certain cardiac patients having a chronic risk for myocardial ischemia because of the chronic potential for an imbalance between the supply of oxygenated blood and the demand for it. Typically, such imbalance occurs during certain stresses, such as exercise, emotional stress or stress associated with a surgical procedure.

Unstable angina – Condition observed in cardiac patients having frequent imbalance between the supply of and the demand for oxygenated blood.

Post-myocardial infarction angina – Condition observed in patients who have recurrent ischemia following a heart attack.

Preconditioning stimuli – Any drug, agent or treatment which induces preconditioning, such as brief hypoxia, adenosine, pinacidil, phorbol ester, A_1 or A_3 adenosine receptor agonists or A_{2a} receptor antagonists.

Myocardial responsiveness – The myocardium can be treated so as to enhance the effectiveness and protective effects of preconditioning. This enhancement leads to a reduction in ischemic damage.

The following methods facilitate the practice of the present invention.

Preparation of Cultured Ventricular Cells

Ventricular cells were cultured from chick embryos 14 days in ovo (Spafas Inc., Storrs, CT) as previously described (16, 23). Cells were cultivated in a humidified 5% CO_2-95% air mixture at 37 C. All experiments were performed on day 3 in culture, at which time cells exhibited rhythmic spontaneous contraction. The medium was changed to a HEPES-buffered medium containing (mM) 139 NaCl, 4.7 KCl, 0.5 $MgCl_2$, 0.9 $CaCl_2$, 5 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) and 2% fetal bovine serum, pH 7.4, 37 C before

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exposing the myocytes to the various conditions at 37 C.
Control myocytes were maintained in HEPES-buffered medium
under room air. Hypoxia and glucose deprivation were
used to induce and simulate ischemia in the cultured

5   cells. Hypoxia was produced by placing the cells in a
hypoxic incubator (NuAire) where O_2 was replaced by N_2 as
previously described (16,23).

Determination of Cell Injury

The extent of hypoxia-induced injury to the

10   ventricular cell was quantitatively determined by the
percentages of cells killed. To quantitate the % of
cells killed, cells were exposed to a trypsin-EDTA Hanks'
balanced salt solution for 10 minutes for detachment
after the various treatment, followed by centrifugation

15   (300 x g for 10 minutes) and resuspension in culture
media for counting in a hemocytometer. Only live cells
sedimented and the cells counted represented those that
survived (46). None of the sedimented cells subsequently
counted included trypan blue. In cells not exposed to

20   hypoxia, trypsin-EDTA treatment followed by re-exposure
to Ca^{2+}-containing culture media did not cause the
appearance of trypan blue-stained cells or any
significant increase in proteins or creatine kinase (CK)
in the trypsin-EDTA media following the 300 x g, 10

25   minute sedimentation of the cells. There was no protein
in the culture media following a second 300 x g
centrifugation of resuspended cells previously treated
with trypsin-EDTA. Thus, trypsin treatment, re-exposure
to Ca^{2+}-containing media or 300 x g sedimentation did not

30   cause any significant damage to the control,
normoxia-exposed cells.

The percentage of cells killed was calculated as the
number of cells obtained from the control group
(representing cells not subjected to any hypoxia or drug

35   treatment) minus the number of cells from the treatment
group divided by number of cells in control group

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multiplied by 100%.

Chemical reagents

2-chloro-N^6-cyclopentyl-adenosine (CCPA) and pinacidil
were purchased from Research Biochemicals International
5   (Natick, MA). Phorbol 12-myristate 13-acetate (PMA) was
purchased from Sigma. 8-(3-chlorostyryl)caffeine (CSC),
N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA)
and 2-chloro-N^6-(3-iodobenzyl)adenosine-
5'-N-methyluronamide (Cl-IB-MECA) were synthesized as
10   previously described (40-42).

The following examples are provided to illustrate
certain embodiments of the invention. They are not
intended to limit the invention in any way.

EXAMPLE I

15   A cultured chick ventricular myocyte model was
developed to investigate the roles of a variety of
compounds known to influence preconditioning. In
accordance with the present invention, it has been
discovered that certain agents, when applied in
20   combination, act synergistically to reduce ischemic
damage to the heart.

Pretreatment of ventricular myocytes with MLA was
assessed to determine whether it enhances the
cardioprotective effect of preconditioning ischemia.
25   Hypoxia (O_2 <1%) and glucose deprivation were employed to
induce and simulate ischemia in the myocyte model of
preconditioning (16, 23, 32).

Cardiac ventricular myocytes were cultured from
chick embryos 14 days in ovo and myocyte injury was
30   induced as described hereinbelow. MLA was added to the
media at the indicated concentrations for a 4 hour period
24 hours prior to 90 minutes of simulated ischemia. The
extent of myocyte injury, determined at the end of the 90
minute simulated ischemia, was assessed by the percentage

of cells killed. The data represent the mean of 4
experiments. At 30 and 300 ng/ml MLA
significantly lowered the percentage of cell death in
response to prolonged ischemia. (One way ANOVA analysis
5 and t test, $p<0.01$).

Exposure of cultured cardiac myocytes to 90 minutes
of prolonged hypoxia causes cell death. Figure 1
illustrates that prior treatment with MLA significantly
reduces cell death relative to the untreated controls.
10 These data are in agreement with the cardioprotective
effect of MLA treatment seen in intact rat, rabbit, and
canine hearts (43-45).

Figure 2 shows that the extent of cardioprotection
induced by a 5 minute period of preconditioning ischemia
15 (induced by hypoxia and the absence of glucose, also
termed simulated ischemia) is enhanced by pretreatment
with MLA. As in Figure 1, cultured myocytes were
pretreated with the indicated concentrations of MLA for 4
hours. Twenty four hours later the cells were subjected
20 to preconditioning ischemia, consisting of 5 minutes of
simulated ischemia. After a 10 minute recovery period in
normal air, the cells were subjected to a prolonged
period (90 minutes) of simulated ischemia, and the
percentage of cells killed was assessed. When myocytes
25 are pretreated with MLA 24 hours prior to preconditioning
ischemia treatment, there is a dose-dependent reduction
in the percentage of cells killed during a prolonged
ischemic event. Thus, pretreatment with MLA in
combination with classic preconditioning ischemia
30 provides a synergistic cardioprotective effect.

EXAMPLE II

Another objective of this study was the elucidation
of the mechanism underlying the observed enhanced
responsiveness of cardiac myocytes to preconditioning
35 stimuli following pretreatment with MLA. The
cardioprotective effects of preconditioning ischemia are
mediated by adenosine receptors and the K_{ATP} channel. The

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following experiments were performed to assess the most
efficacious combination of agents for the prevention of
ischemic damage to the heart.

     As shown in Figure 3A, prior treatment with MLA

5   enhanced the ability of adenosine to elicit the
preconditioning response. In this experiment, myocytes
were treated with either the vehicle (0.04% propylene
glycol, 0.01% ethyl alcohol) or MLA 24 hours prior to a 5
minute exposure to 10 μM adenosine. Adenosine was

10   removed by replacement of the culture medium with
adenosine-free medium, and the cells were exposed to
normal room air O_2 for 10 minutes, followed by 90 minutes
of simulated ischemia treatment. The sequential
treatment of myocytes with MLA followed by adenosine

15   significantly decreased the percentage of myocytes killed
by prolonged ischemia, as compared to pretreatment with
the vehicle followed by exposure to adenosine.
Similarly, Figure 3B and 3C show that prior treatment of
myocytes with MLA followed by adenosine agonist, CCPA or

20   by A3 agonist, IB-MECA, as a classical preconditioning
stimuli, also significantly reduced the percentage of
myocytes killed by prolonged ischemia as compared to pre-
treatment with the vehicle followed by exposure to CCPA
or IB-MECA alone, respectively.

25   An enhanced adenosine-mediated classical
preconditioning response in myocytes pre-exposed to MLA
suggests that MLA acts to increase the responsiveness of
myocytes to the preconditioning effect of adenosine.
Since the adenosine receptor mediates the preconditioning

30   response to adenosine, such results may further suggest
that MLA activates or pre-activates the adenosine
receptor/G-protein/PKC/K_{ATP} channel pathway. Consistent
with this hypothesis, the ability of the phorbol ester
phorbol 12-myristate 13-acetate (PMA) to mediate a

35   preconditioning response was also enhanced by
pretreatment with MLA. See Figure 4. This enhancement
by 300 ng/ml MLA pretreatment was significant over a

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range of PMA concentrations from 0.01 μM to 1 μM. The
preconditioning effects of CCPA or IB-MECA were also
enhanced by pretreatment of myocytes with MLA. This
enhancement was not observed in myocytes pretreated with
5   vehicle alone (data not shown).

MLA pretreatment also enhanced the preconditioning
response mediated by pinacidil. See Figure 5. The data
are consistent with the notion that MLA can pre-activate
the adenosine receptor, G protein, PKC, the K_{ATP} channel,
10 or all four molecules. On the other hand, as the K_{ATP}
channel is the most distal effector of the four, MLA may
pre-activate or activate the K_{ATP} channel directly to
render the cardiac myocyte more responsive to the
preconditioning effect of the K_{ATP} channel opener
15 pinacidil.

EXAMPLE III

The experiments described in the previous examples
suggest that MLA acts to enhance the preconditioning
20 effects of various pharmacological agents such as
adenosine and pinacidil.

The synergistic effects obtained using a combination
of MLA and other preconditioning stimuli prompted studies
assessing the activity of agonists and antagonists of
25 adenosine receptors following pretreatment with MLA.

The experimental protocol utilized in these studies
entailed pretreatment of myocytes with MLA for a 4 hour
period 24 hours prior to exposure of the cardiac myocytes
to 90 minutes of simulated ischemia. During this 90
30 minute simulated ischemia period, several
cardioprotective agents were administered individually to
cells and their protective effects were assessed.

Figure 6 illustrates that prior treatment of
myocytes with MLA enhanced adenosine A_1 agonist mediated
35 protection during the 90 minute exposure to ischemia. In
MLA pretreated myocytes, the presence of CCPA during the
90 minute ischemia resulted in a greater reduction in the

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number of cells killed as compared to the number of cells killed following pretreatment with vehicle alone. Again the synergistic effects mediated by the two cardioprotective agents are illustrated by the data.

5   Similar results were obtained when cells were pretreated with MLA then treated with IB-MECA, an A_3 adenosine receptor agonist during the 90 minute ischemia as described above for Figure 6. See Figure 7. Again the results showed a synergistic cardioprotective effect

10   mediated by the combination of the two agents.

Figure 8 shows the results obtained by pretreatment with MLA followed 24 hours later, by exposure to pinacidil, a K_{ATP} channel opener, during the 90 minute ischemia. In MLA-pretreated myocytes, pinacidil caused a

15   greater reduction in the number of cells killed as compared to vehicle pretreatment.

Additional experiments were performed to assess the differences in cardioprotection observed when MLA was administered as the second cardioprotective agent and an

20   adenosine agonist, KATP channel opener or protein kinase C activator was administered as the first cardioprotective agent. The experiments were performed as follows: Cardiac myocytes were exposed to the first cardioprotective agent for 5 minutes and the agent was

25   removed. Cells were then exposed to a period of simulated ischemia in the presence or absence of 300 ng/ml of MLA. The percentage of cells killed by simulated ischemia alone was 27 ± 2% (n=9) in the absence of any treatment with any cardioprotective agent. When

30   MLA was present during the simulated ischemia, the percentage of cells killed was reduced from 27 ±2% to 14 ±1.2%. When cells were exposed to 5 minutes of 10 μM pinacidil, a KATP channel opener followed by exposure to simulated ischemia (in the absence of MLA), the

35   percentage of cells killed was 12.3 ± 1. When the pinacidil-treated cells were exposed to simulated ischemia in the presence of MLA, the percentage of cells

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killed was reduced from 12.3 ± 1% to 6.3 ± 1.2% (n=3).
When cells were exposed to 5 minutes of 1 µM PMA, a PKC
activator, followed by exposure to simulated ischemia
(in the absence of MLA), the percentage of cells killed

5   was 14 ± 1. When PMA-treated cells were then exposed to
simulated ischemia in the presence of MLA, the percentage
of cells killed was reduced to 10.5 ± 1 (n=3). When cells
were exposed to 5 minutes of 10 µM adenosine, followed by
exposure to simulated ischemia (in the absence of MLA),

10   the percentage of cells killed was 12 ± 1.4%. When
adenosine treated cells were then exposed to simulated
ischemia, in the presence of MLA, the percentage of cells
killed was reduced to 7.4 ± 1.2% These results indicate
that the cardioprotective agents of the invention are

15   effective in reducing ischemic damage of the heart
regardless of the order in which they are administered to
cells.

       The data presented in the Figures illustrate the
synergistic effects mediated by the combination of MLA,

20   adenosine receptor agonists and K_{ATP} channel openers in
mediating cardioprotection.   Accordingly, other agents
known to act on the adenosine receptors are contemplated
for use in the present invention. Earlier studies have
revealed that antagonists at the A_{2a} receptor mediate

25   cardioprotecctive effects. Thus, the combined use of MLA
or analogs thereof followed by treatment with an A_{2a}
antagonist should also prove to be efficacious in
reducing ischemic injury of the heart. Candidate
compounds of the following formula that bind and inhibit

30   adenosine A_{2a} receptor activation are set forth below in
Table I.

14
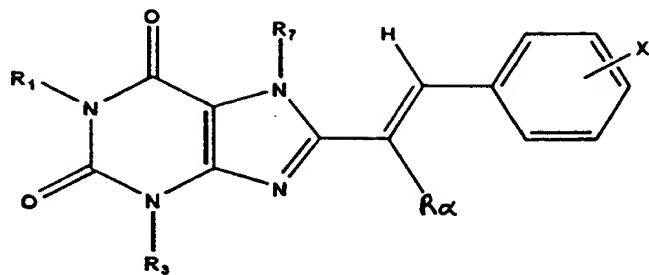
R1, R3 = methyl, ethyl, propyl, allyl

R7 = H, alkyl (C1-C8)

Rα = H

TABLE I
Affinities of 8 Styrylxanthine Derivatives in Radioligand Binding Assays at Rat Brain A_1 and A_2-Receptors (47)

	cmpd	$R_1 \cdot R_3$	R_7	X	A_1/A_2 ratio
5	15b	Me	Me	H	41
	17b	Me	Me	2-MeO	18
	19b	Me	Me	3-MeO	64
	20b	Me	Me	3-CF$_3$	25
	21b	Me	Me	3-NO$_2$	11
10	22b	Me	Me	3-NH$_2$	30
	23	Me	Me	3-(AcNH)	240
	24	Me	Me	3-(HOOC(CH$_2$)$_2$CONH)	250
	25	Me	Me	3-(t-BOC-NH)	30
	26	Me	Me	3-[t-BOC)$_2$N]	15
15	27b	Me	Me	3-F	190
	28	Me	Me	3-Cl	520
	29b	Me	Me	4-MeO	44
	32b	Me	Me	3,4-(MeO)$_2$	70
	33a	Me	H	3,5-(MeO)$_2$	25
20	33b	Me	Me	3,5-(MeO)$_2$	>200
	34b	Me	Me	3,5-F$_2$	230
	35	Me	Me	3,5-(MeO)$_2$-4-OH	19
	36	Me	Me	4-AcO-3,5-(MeO)$_2$	93
	37	Me	Me	4-(4-PhCH$_2$O)-3,5-(MeO)$_2$	30
25	38	Me	Me	4-(4-NH$_2$-BuO)-3,5 (MeO)$_2$	36
	39	Me	Me	4-[4-(tBOC-NH)BuO]-3,5-(MeO)$_2$	42
	40	Me	Me	4-(4-NH$_2$-trans-CH$_2$CH= CHCH$_2$O-3,5-(MeO)$_2$	28
	41	Me	Me	4-(4-AcNH-trans-CH$_2$CH= CHCH$_2$O)-3,5-(MeO)$_2$	>50
	42	Me	Me	4-(4-t-BOC-NH-trans-CH$_2$CH= CHCH$_2$O-3,5-(MeO)$_2$	>40
30	43b	Me	Me	2,3,4-(MeO)$_3$	34
	44b	Me	Me	3,4,5-(MeO)$_3$	70 [>5600]
	45b	Et	Me	3,4,5-(MeO)$_3$	34
	46	allyl	Me	3,4,5-(MeO)$_3$	13 [>6700]
	51b	Pr	Me	3-Cl	14
35	52b	Pr	Me	3,4-(MeO)$_2$	19 [190]
	53b	Pr	Me	3,5-(MeO)$_2$	110

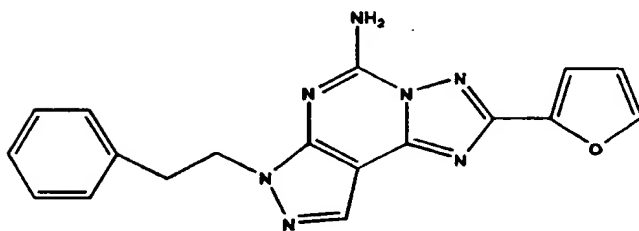## Additional compounds contemplated for use as A_{2a}

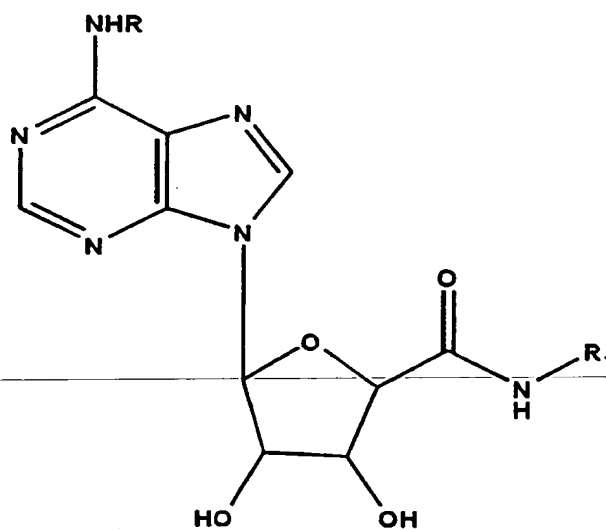antagonists include:
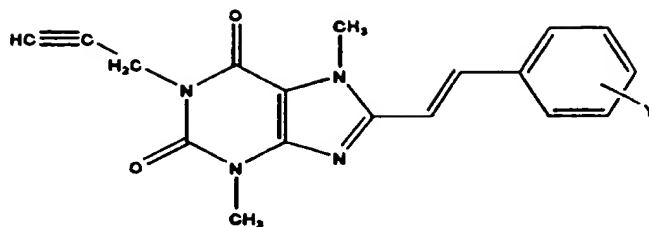
Y = m-Br or p-SO₃H   (DMPX derivative)
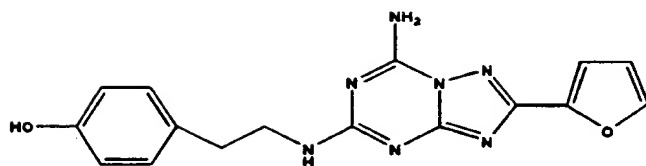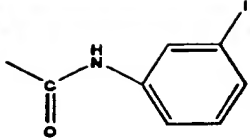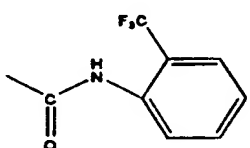

5   (ZM241385)
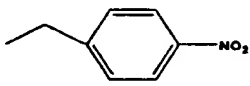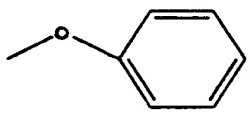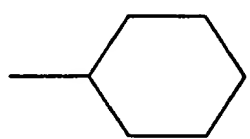

(SCH58261)

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As mentioned previously, agonists at the A_1 and A_3 adenosine receptors are also contemplated for use in the methods of the present invention. Suitable agonists of the following formula are set forth below in Table II.

5

| COMPOUND (Reference)	R_1	R	A_1 (nm)	A_{2a} (nm)	A_3 (nm)
Compound 4q (Reference 27)	Ethyl	(3-iodophenyl acetamido structure)	16	3940	30
Compound 4d (Reference 27)	Ethyl	(2-trifluoromethyl F_3C acetamido structure)	384	>10,000	54
Compound 37 (Reference 8)	Ethyl	(4-ethyl nitrobenzyl structure, NO_2)	49	574	9.0
Compound 11 (Reference 28)	Methyl	(O-methoxy phenyl structure)	2060	66,300	1340
N^6-cyclohexyl NECA (Reference 29)	Ethyl	(cyclohexyl structure)	0.43	170	16

$4q = N^6-((3-iodophenyl)carbamoyl)adenosine -5'uronamide$

$4d = N^6-((2-trifluoromethyl)carbamoyl)adenosine-5'uronamide$

compound $37 = N^6-(4-Nitrobenzyl)adenosine-5'-N-ethyluronamide$

compound 11 = 6-(O-Phenylhydroxylamino)purine-9-beta-ribofuranoside-5'-N-methyluronamide

N6-cyclohexyl NECA = N6-cyclohexyl 5'-N-ethylcarboxamidoadenosine

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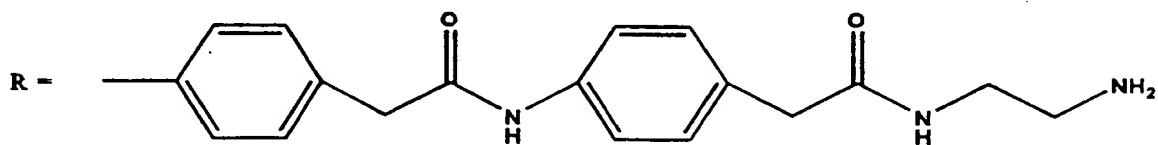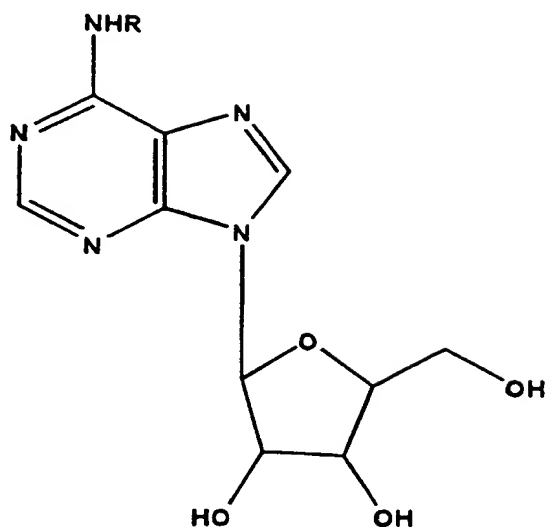
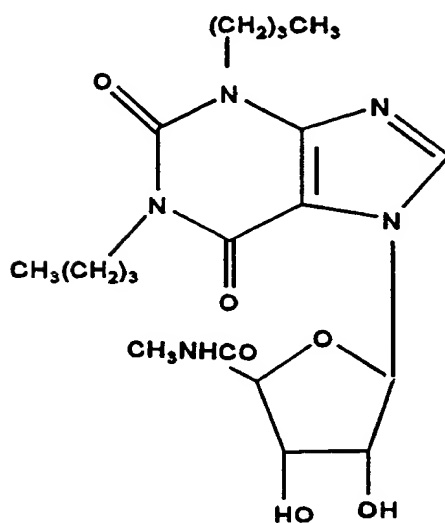| COMPOUND	R	A_1 (nm)	A_2 (nm)	A_3 (nm)
Compound 8*	(See immediately above.)	0.85	210	4

*N^6-[4-[[[4-[2-aminoethyl)amino]carbonyl]methyl]-
anilino]carbonyl]methyl]phenyl]adenosine

20

Other effective A3 agonists suitable for use in
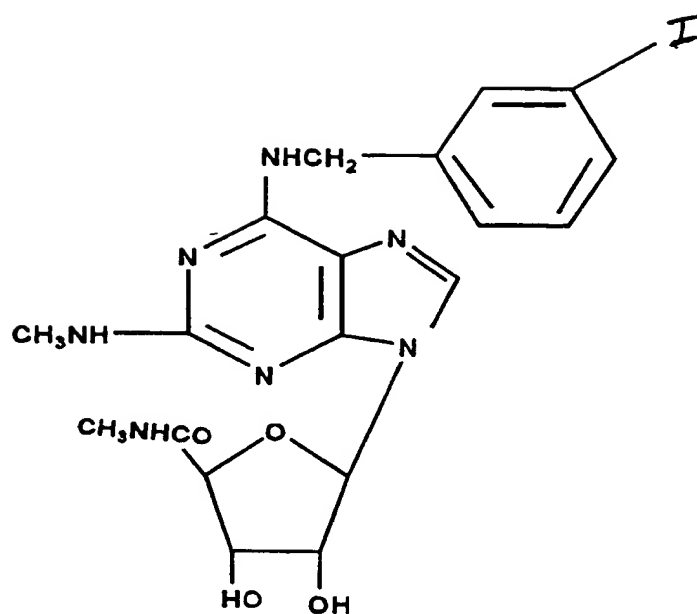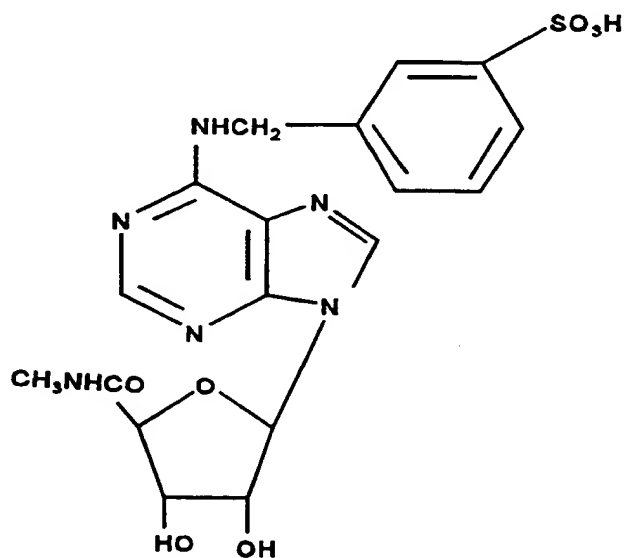practicing the methods of the present invention are set
forth below:

5   MRS 584

MRS 479 (48)

21
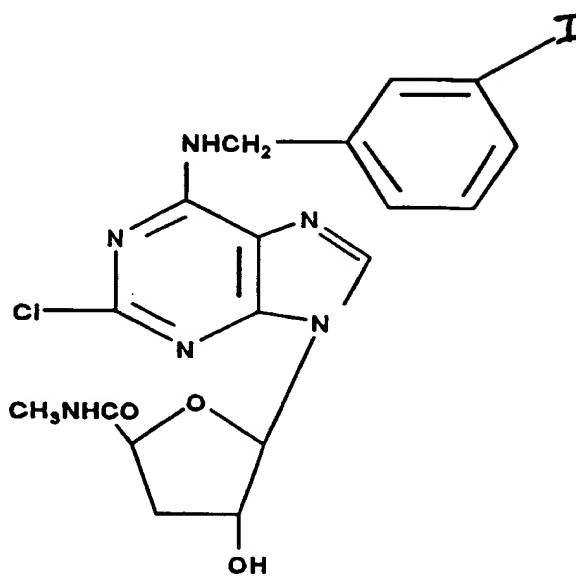
MRS 537 (13)

MRS 1340 (8)

22

EXAMPLE IV

The synergistic cardioprotective effect of MLA used in combination with A_3/A_1 agonists and a K_{ATP} channel opener has been demonstrated herein. Combinations of
5   these agents may be used therapeutically in patients who suffer from ischemic damage due to stable angina, unstable angina or post-myocardial infarction angina.

Several administration modalities may be utilized to treat patients with the agents of the invention. These
10   modalities are influenced by bioavailability factors. For example, if the compound is metabolized in the liver or excreted in the bile, some of the active compound absorbed from the gastrointestinal tract will be inactivated by the liver before it can reach the general
15   circulation and for distribution to the site of action. It is not believed that adenosine A_1/A_3 receptor agonists, adenosine A_{2a} receptor antagonists or K_{ATP} will be subject to this first pass loss. Additionally, because the agonists of the invention are polar and water soluble, it
20   is expected that they will have a small volume of distribution, and thus be readily eliminated by the kidney. Moreover, binding of these agents to plasma proteins may limit their free concentrations in tissues and at their locus of action since it is only the unbound
25   drug which equilibrates across membrane receptor sites.

Another factor affecting bioavailability is the distribution of the agonists to tissues. Given the relatively small size of these compounds and their water solubility, it is anticipated that the compounds will
30   have a relatively fast second phase of drug distribution. This distribution is determined by both the blood flow to the particular tissue of the organ such as the heart, as well as the rate at which the compounds diffuse into the interstitial compartment from the general circulation
35   through the highly permeable capillary endothelium.

23

MLA or synthetic analogs thereof are lipophilic and will be administered intravenously in a 10% ethanol, 40% propylene glycol water solution. Alternatively MLA or its analogs can be given orally in capsules or tablets.

5   MLA or its analogs would be administered in doses ranging from 1-20 μg/kg body weight. Patients may be perfused with the adenosine receptor A_1 or A_3 agonists, adenosine A_{2a} receptor antagonists or K_{ATP} channel openers of the invention by dissolving them in normal saline solution or

10   using emulsifying agents or cosolvents followed by intravenous administration every four to six hours. Effective doses usually range from 100 to 300 nM. For example, considering a 15 liter volume of distribution for a 70 kg patient, a loading dose ranging from 0.5 to

15   1.5 mg is preferably used. Depending on the half-life of the agonists in the body, several doses, e.g., 1.5-4.5 mg may be administered per day.

     Alternatively, a time-release or slow-release preparation may be utilized which allows for periodic or

20   constant release of the agonists, antagonists or channel openers over a given time period. This method would allow for a single dose of these agents in a given day. Methods for preparing such capsules are well known to those of skill in the art of drug delivery.

25   While the combined use of MLA with specific A_3 and A_1 agonists or K_{ATP} channel openers has been exemplified herein, other compounds have been identified which have high affinity for both the A_3 and A_1 receptor, also other compounds are known to those skilled in the art that

30   function to open the K_{ATP} channel. Additionally, analogs or derivatives of MLA are known. Any of these compounds or agents therefore may also be used in the practice of the instant invention.

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While certain preferred embodiments of the present

20   invention have been described and specifically

exemplified above, it is not intended that the invention

be limited to such embodiments. Various modifications

may be made to the invention without departing from the

scope and spirit thereof as set forth in the following

25   claims.

29

What is claimed is:

    1.   A method for preventing or reducing ischemic
heart damage, in a patient in need of such treatment,
comprising administering to said patient a first
    5 cardioprotective agent followed by adminstration of a
second cardioprotective agent, said first and second
cardioprotective agents acting synergistically to protect
the heart from ischemic damage.

    2.   A method as claimed in claim 1, wherein said
    10 first cardioprotective agent is selected from the group
consisting of monophosphoryl lipid A, a synthetic analog
of monophosphoryl lipid A, adenosine A1 agonists or
adenosine A3 agonists.

    3.   A method as claimed in claim 1, wherein said
    15 second cardioprotective agent is selected from the group
consisting of adenosine A1 receptor agonists, adenosine
A3 receptor agonists, agonists listed in Table II of the
present specification, MRS 584, MRS 479, MRS 537, MRS
1340, adenosine A2a receptor antagonists, antagonists
    20 listed in Table I of the present specification, PKC
activators and K_{ATP} channel openers.

    4.   A method as claimed in claim 1, wherein said
first cardioprotective agent is administered to said
patient by an administration means selected from the
    25 group consisting of intravenous administration, direct
cardiac perfusion, or oral administration.

    5.   A method as claimed in claim 1, wherein said
second cardioprotective agent is administered to said
patient by an administration means selected from the
    30 group consisting of intravenous administration, direct
cardiac perfusion, or oral administration.

6.   A method as claimed in claim 1, wherein said
patient is in need of said treatment due to the presence
of angina selected from the group consisting of unstable
5   angina or post-myocardial infarction angina.

7.   A method as claimed in claim 1, wherein said
cardioprotective agents are admininistered to said
patient prior to a surgical procedure which may cause
cardiac ischemic damage.

10   8.   A method as claimed in claim 1, wherein said
cardioprotective agents are admininistered to said
patient during a surgical procedure which may cause
cardiac ischemic damage.

9.   A method as claimed in claim 1, wherein said
15   cardioprotective agents are admininistered to said
patient following a surgical procedure which may cause
cardiac ischemic damage.

10.   A method for enhancing myocardial
responsiveness to preconditioning stimuli in a patient in
20   need of such treatment comprising administering to said
patient a first cardioprotective agent followed by
adminstration of a second cardioprotective agent, said
first and second cardioprotective agents acting
synergistically to enhance myocardial responsiveness.

25   11.   A method as claimed in claim 10, wherein said
first cardioprotective agent is selected from the group
consisting of monophosphoryl lipid A, a synthetic analog
of monophosphoryl lipid A, adenosine A1 agonists or
adenosine A3 agonists.

30   12.   A method as claimed in claim 10, wherein said
second cardioprotective agent is selected from the group

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consisting of adenosine A1 receptor agonists, adenosine
A3 receptor agonists, adenosine A2a receptor antagonists,
antagonists listed in Table I of the present
specification, PKC activators and K_{ATP} channel openers.

5   13.   A method as claimed in claim 10, wherein said
first cardioprotective agent is administered to said
patient by an administration means selected from the
group consisting of intravenous administration, direct
cardiac perfusion, or oral administration.

10   14.   A method as claimed in claim 10, wherein said
second cardioprotective agent is administered to said
patient by an administration means selected from the
group consisting of intravenous administration, direct
cardiac perfusion, or oral administration.

15

15.   A method as claimed in claim 10, wherein said
patient is in need of said treatment due to the presence
of angina selected from the group consisting of unstable
angina or post-myocardial infarction angina.

20   16.   A method as claimed in claim 10, wherein said
cardioprotective agents are admininistered to said
patient prior to a surgical procedure which may cause
cardiac ischemic damage.

17.   A method for reducing or preventing ischemic
25   heart damage in a patient in need of such treatment,
comprising the administration of first and second
cardioprotective agents, said first cardioprotective
agent being selected from the group consisting of A1
adenosine agonists or A3 adenosine agonists, K_{ATP} channel
30   openers, and PKC activators followed by the
administration of a second cardioprotective agent
selected from the group consisting of monophosphoryl
lipid A or synthetic analogs of monophosphoryl lipid A.

32

18.   A method as claimed in claim 17, wherein said cardioprotective agents are admininistered to said patient prior to a surgical procedure which may cause cardiac ischemic damage.

5   19.   A method as claimed in claim 17, wherein said cardioprotective agents are admininistered to said patient during a surgical procedure which may cause cardiac ischemic damage.

20.   A method as claimed in claim 17, wherein said
10   cardioprotective agents are admininistered to said patient following a surgical procedure which may cause cardiac ischemic damage.

21.   A method as claimed in claim 17, wherein said first cardioprotective agent is administered to said
15   patient by an administration means selected from the group consisting of intravenous administration, direct cardiac perfusion, or oral administration.

22.   A method as claimed in claim 17, wherein said second cardioprotective agent is administered to said
20   patient by an administration means selected from the group consisting of intravenous administration, direct cardiac perfusion, or oral administration.

23.   A method as claimed in claim 17, wherein said
25   patient is in need of said treatment due to the presence of angina selected from the group consisting of unstable angina or post-myocardial infarction angina.

24.   A method as claimed in claim 17, wherein said K_{ATP} channel opener is nicorandil.

30   25.   A method as claimed in claim 17, wherein said first cardioprotective agent is selected from the group

33

consisting of agonists listed in Table II of the present
specification, MRS 584, MRS 479, MRS 537, and MRS 1340.

26.   A method for enhancing myocardial
responsiveness to preconditioning stimuli in a patient in
5   need of such treatment comprising administering to said
patient a first cardioprotective agent followed by
adminstration of a second cardioprotective agent, said
first cardioprotective agent being selected from the
group consisting of A1 adenosine agonists or A3 adenosine
10 agonists, K_{ATP} channel openers, and PKC activators
followed by the administration of a second
cardioprotective agent selected from the group consisting
of A2a antagonists, monophosphoryl lipid A or synthetic
analogs of monophosphoryl lipid A, said first and second
15 cardioprotective agents acting synergistically to enhance
myocardial responsiveness.

27.   A method as claimed in claim 26, wherein said
first cardioprotective agent is administered to said
patient by an administration means selected from the
20 group consisting of intravenous administration, direct
cardiac perfusion, or oral administration.

28.   A method as claimed in claim 26, wherein said
second cardioprotective agent is administered to said
patient by an administration means selected from the
25 group consisting of intravenous administration, direct
cardiac perfusion, or oral administration.

29.   A method as claimed in claim 26, wherein said
patient is in need of said treatment due to the presence
30 of angina selected from the group consisting of unstable
angina or post-myocardial infarction angina.

30.   A method as claimed in claim 26, wherein said
cardioprotective agents are admininistered to said

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patient prior to a surgical procedure which may cause
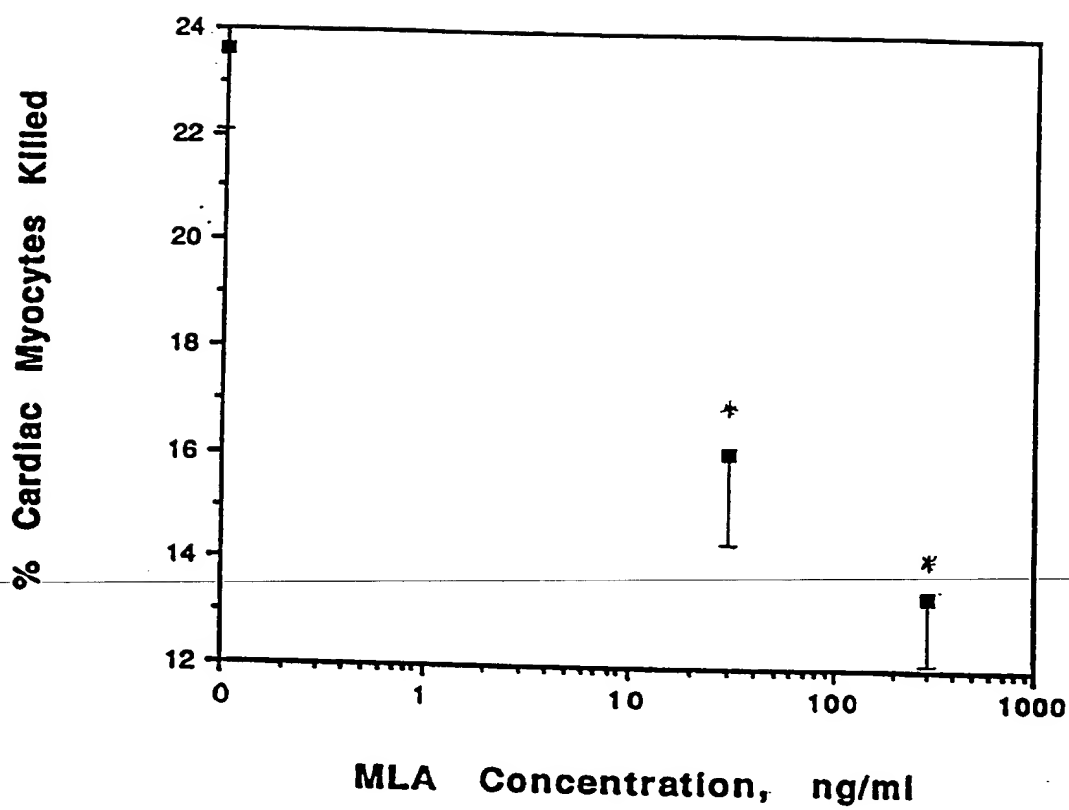cardiac ischemic damage.

Figure 1

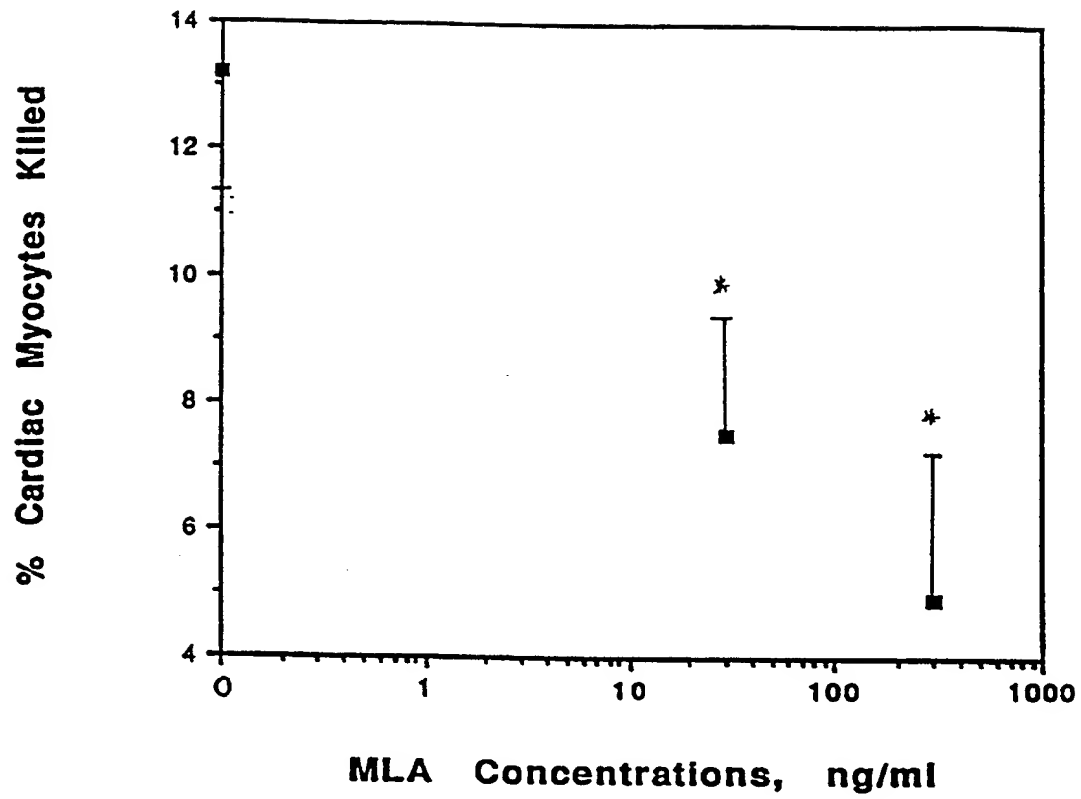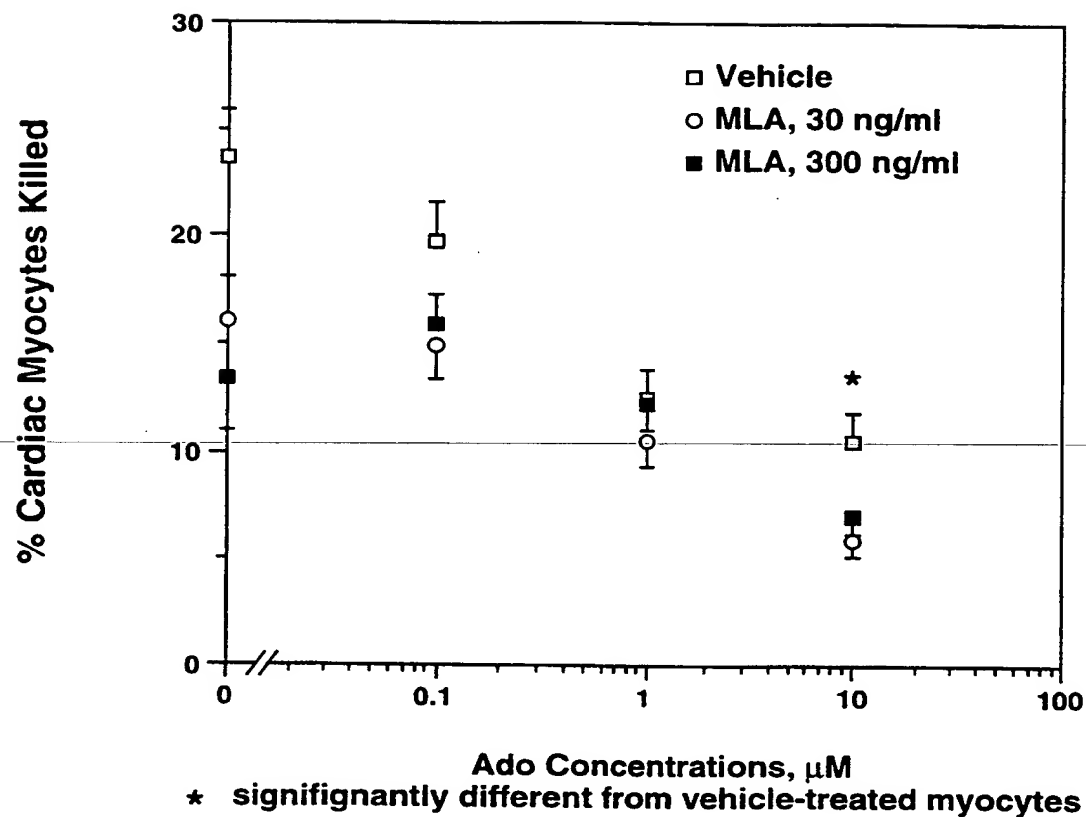Figure 2

Fig. 3A

Fig. 3B

Fig. 3C
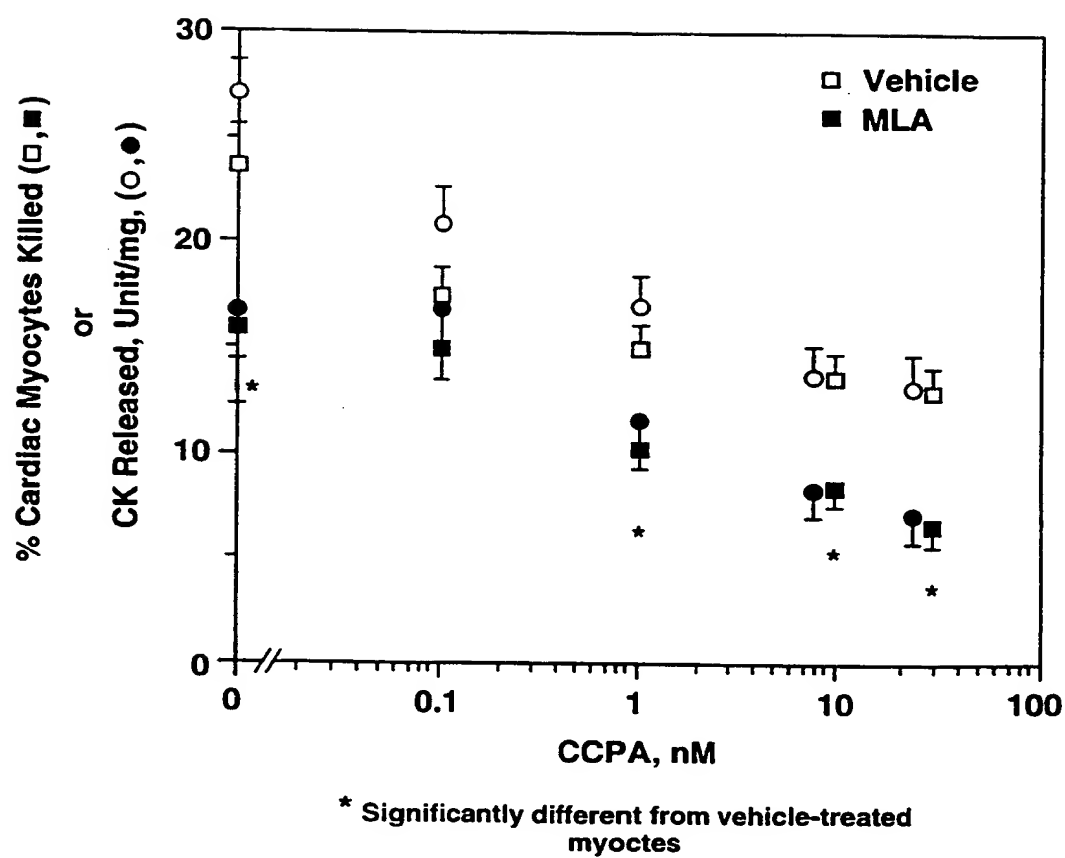
Figure 4

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Figure 5

Figure 6

Figure 7

BNSDOCID: <WO___9920284A1_I_>
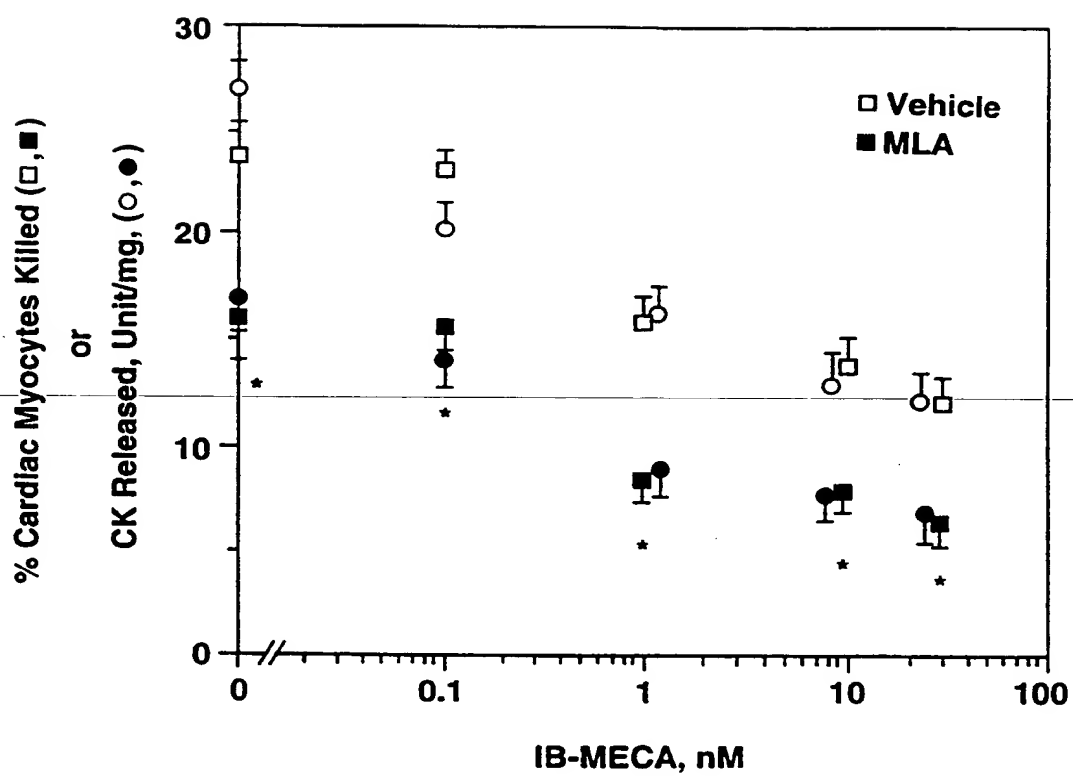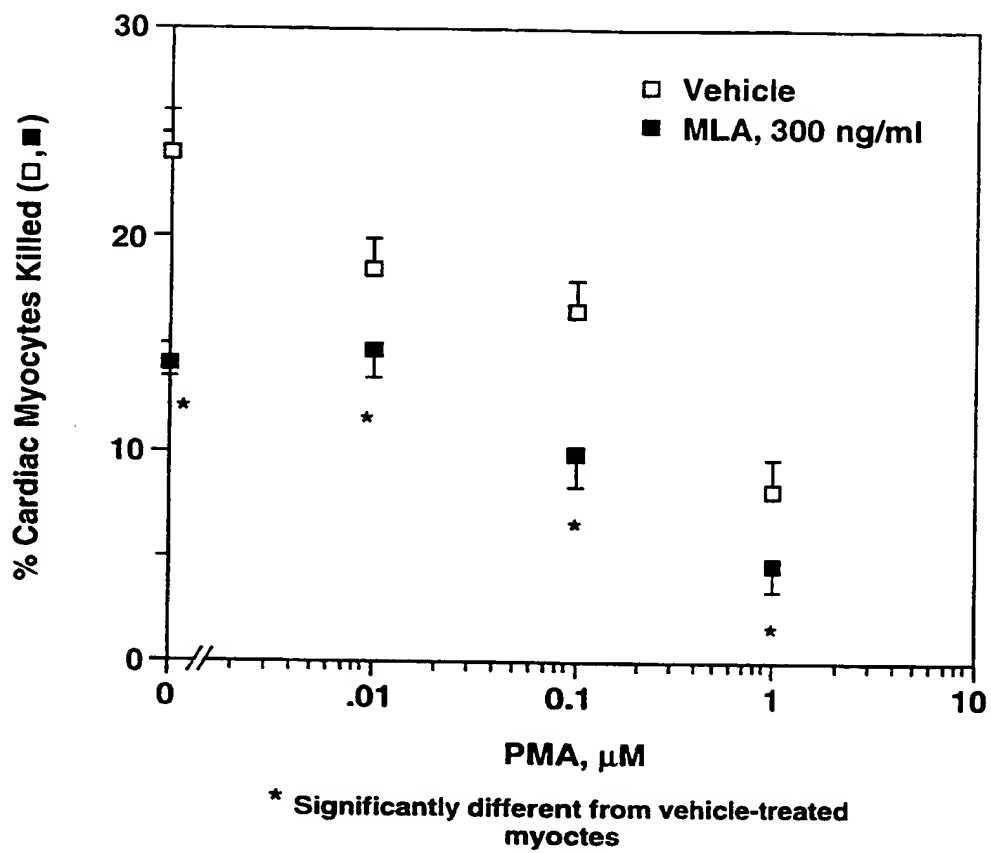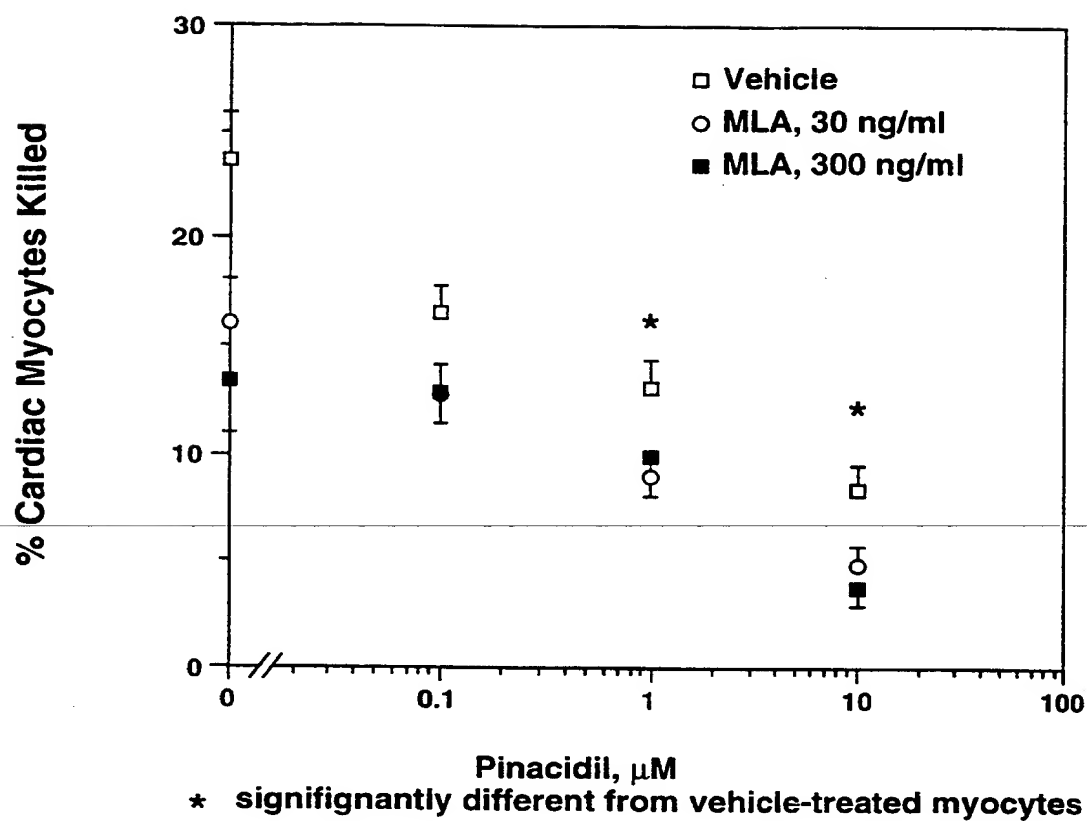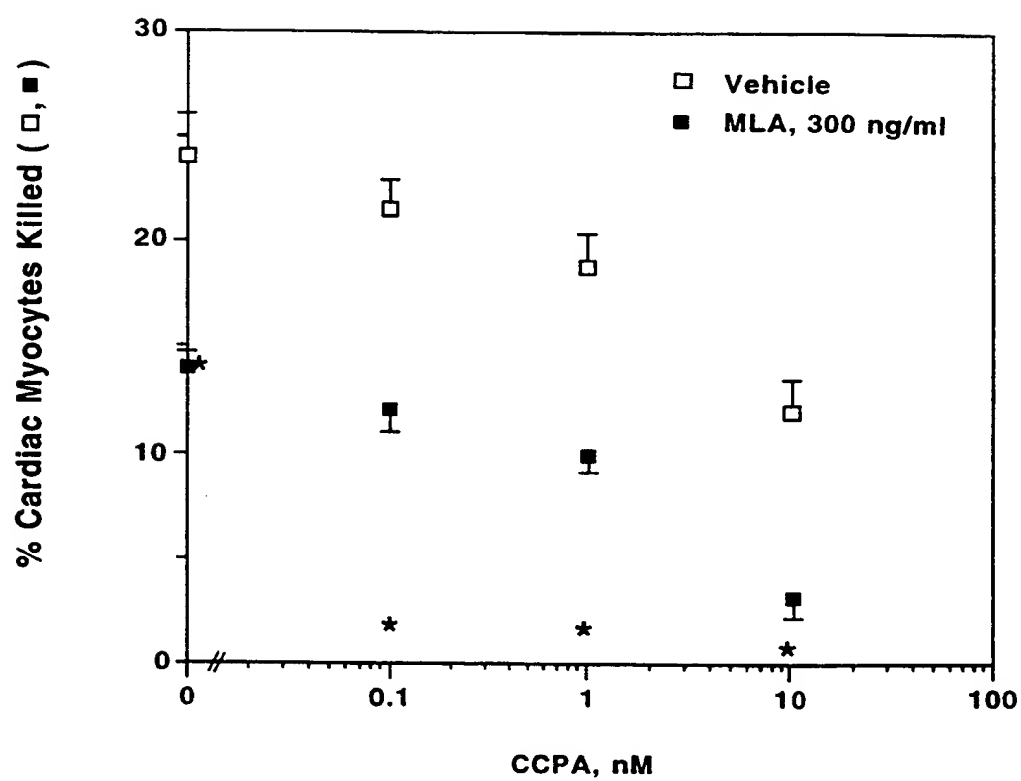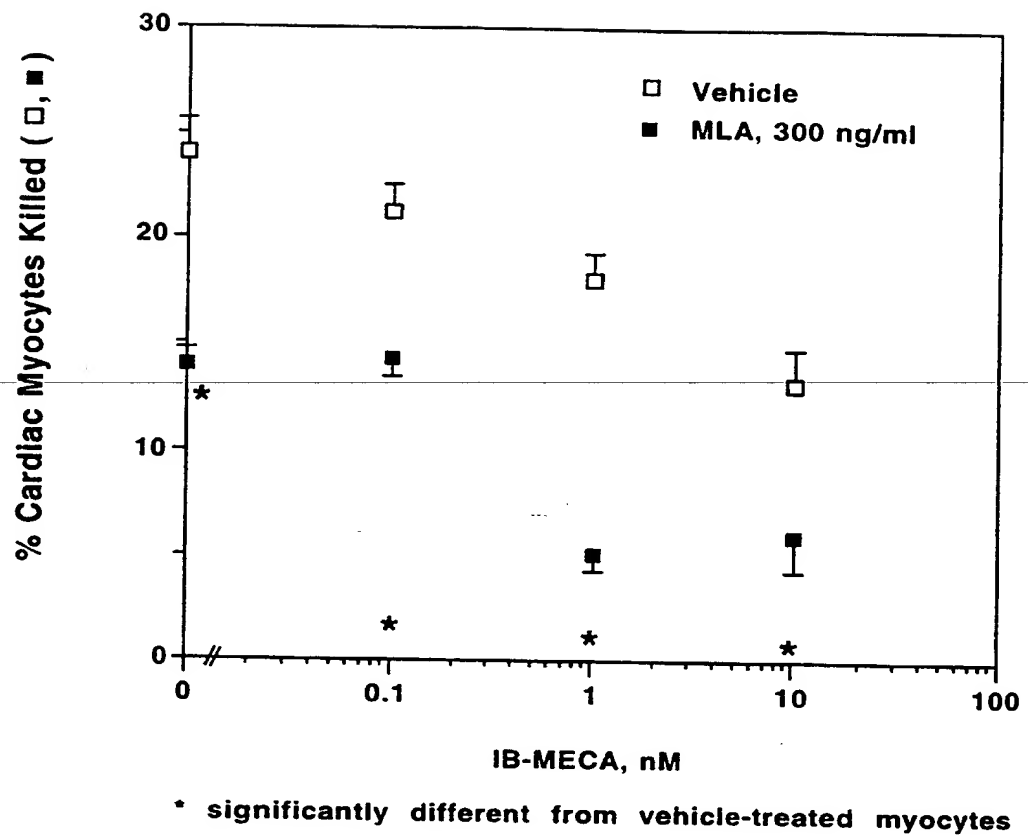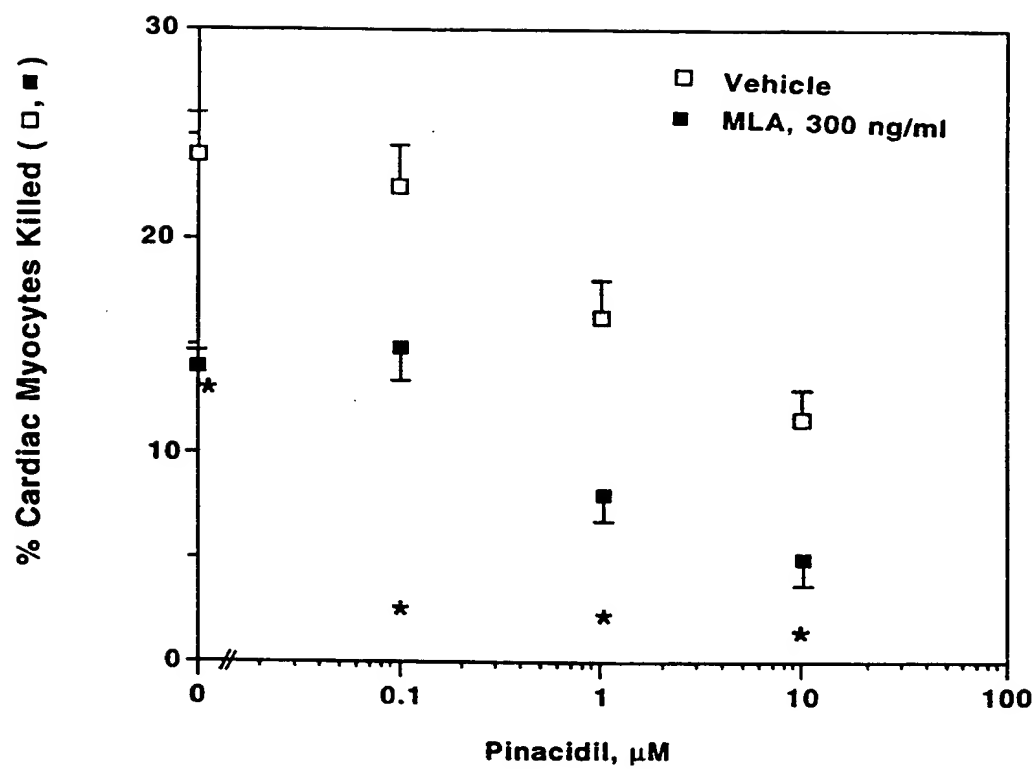
Figure 8

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/715, 31/44

US CL :514/54, 353, 355, 356, 921

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/54, 353, 355, 356, 921

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS-ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,870,090 A (NODA et al.) 26 September 1989, see the entire document.	1, 3, 5-10, 12, 14-24, 26, 28-30
Y	US 5,286,718 A (ELLIOTT) 15 February 1994, see the entire document.	1, 2, 4, 6-11, 13, 15-21, 23, 25-27, 29 and 30
Y	US 5,354,764 A (GROVER et al.) 11 October 1994, see the entire document.	1, 2, 4, 6-11, 13, 15-21, 23, 25-27, 29 and 30

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X" —	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 FEBRUARY 1999	**19 FEB 1999**

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	KEVIN E. WEDDINGTON
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235

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